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Microchip capillary gel electrophoresis using programmed field strength gradients for the ultra-fast analysis of genetically modified organisms in soybeans

Yun-Jeong Kim [a], Joon-Seok Chae [b], Jun Keun Chang [c], Seong Ho Kang [a,*]

[a] *Department of Chemistry, Chonbuk National University, Jeonju 561-756, South Korea*
[b] *Bio-safety Research Institute and College of Veterinary Medicine, Chonbuk National University, Jeonju 561-756, South Korea*
[c] *Digital Bio Technology, SKC Central Research Institute Room 511, Suwon 440-301, South Korea*

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Abstract

We have developed a novel method for the ultra-fast analysis of genetically modified organisms (GMOs) in soybeans by microchip capillary gel electrophoresis (MCGE) using programmed field strength gradients (PFSG) in a conventional glass double-T microchip. Under the programmed electric field strength and 0.3% poly(ethylene oxide) sieving matrix, the GMO in soybeans was analyzed within only 11 s of the microchip. The MCGE-PFSG method was a program that changes the electric field strength during GMO analysis, and was also applied to the ultra-fast analysis of PCR products. Compared to MCGE using a conventional and constantly applied electric field, the MCGE-PFSG analysis generated faster results without the loss of resolving power and reproducibility for specific DNA fragments (100- and 250-bp DNA) of GM-soybeans. The MCGE-PFSG technique may prove to be a new tool in the GMO analysis due to its speed, simplicity, and high efficiency.

Keywords: Chip technology; GMO; Fast analysis; Programmed field strength gradients

1. Introduction

Biotechnology or gene transfer enables us to use important genes or characteristics from one organism and to place the genes with advantageous characteristics into another species. Transgenesis refers to the transfer of a gene or genes from one species into another host species. The newly introduced genes encode a new protein that gives the desired qualities in the host plant, animal or bacteria. Generally, this genetically modified organism (GMO) expresses new specific properties such as herbicide tolerance, insect resistance and productivity increase [1,2]. While the demand for commercial use of GMO has continuously increased due to its multiple advantages such as agricultural productivity, there is still a lot of controversy about GMO due to its potential risks to human health and world ecology. Thus, both European and Japanese legislation have recently introduced requirement of the obligatory labeling of foodstuffs produced from GMOs with a threshold of 1 and 5%, respectively, of GM material in a non-GM background [3–6]. Therefore, accurate and fast analytical methods for the quantification of GMOs in foodstuffs and/or for products containing GMO are required.

There are various detection methods for the identification of GMOs including protein-based methods, SDS gel electrophoresis, Western blot analysis, enzyme-linked immunosorbant assay (ELISA) [7,8], nucleotide-base amplification methods [9], detection of specific promoter and terminator sequences [10], capillary electrophoresis (CE) [11,12] and real-time PCR [9]. Among these techniques, slab gel electrophoresis, CE and real-time PCR method are most commonly used for the detection of GMO-specific amplification products. CE exhibits a faster separation with

* Corresponding author. Tel.: +82 63 270 3421; fax: +82 63 270 3408.
E-mail address: shkang@chonbuk.ac.kr (S.H. Kang).

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high resolution than the slab gel electrophoresis [11,12] and real-time PCR provides a quantitative analysis of GMO [9]. However, commercially available CE and real-time PCR systems are expensive.

Since the first demonstrations by Manz et al. [13] and Harrison et al. [14], microchip capillary electrophoresis (MCE) is fast becoming an important technique for the analysis of DNA fragments because of the analytical throughput, speed, small reagent volume, automation, miniaturization and high resolution [15–18]. One of the most significant advantages of MCE in DNA fragment analysis is its high speed compared to the traditional slab gel electrophoresis and CE. McDowell et al. reported that MCE was a fast and accurate alternative to PCR product quantification compared to the traditional slab gel electrophoresis method [23]. However, a major limitation of DNA fragment analysis by MCE is the use of a sieving matrix for the gel electrophoretic separation of DNA fragments. Because DNA fragment separation depends on the DNA size in the MCE separation, the separation of specific size DNA molecules does not easily acquire a short separation time without the loss of resolving power under constant electric field strength.

We recently reported the possibility of the fast separation method of DNA fragments by microchip capillary gel electrophoresis (MCGE) using programmed field strength gradients (PFSG) [19]. The PFSG allowed the fast separation of, and enhanced resolving power for target DNA fragments of long size (>1000 bp DNA). The method does not involve special requirements and/or devices. The results of MCGE-PFSG are based on electric field strength gradients that use a MCGE separation step in a sieving gel matrix. In this study, we have established a strategy for the ultra-fast analysis of GMO in soybeans, which have a relative short DNA fragment size (i.e., 100- and 250-bp DNA), by MCGE-PFSG in a microchip. The PFSG technique was able to decrease the analysis time for the detection of the CaMV 35S promoter sequence that is present in most GMOs [20]. The lectin, an endogenous and plant-specific gene, is also detected to prove that the extracted DNA from soybean. This paper also shows the ME-PFSG technique can be used for the ultra-fast analysis of all DNA fragments without considering the DNA fragment size.

2. Experimental

2.1. Chemical and reagents

1× TBE buffer (0.089 M Tris, 0.089 M borate and 0.002 M EDTA, pH 8.3) was prepared by dissolving pre-mixed powder (Amerosco, Solon, OH, USA) in deionized water. The dynamic coating matrix of the microchip was made by dissolving 0.5% (w/v) of M_r 1,000,000 polyvinylpyrrolidone (PVP) (Polyscience, Warrington, England) into the 1× TBE buffer with 0.5 μg/ml ethidium bromide (EtBr) (Sigma, St. Louis, MO, USA). The mixture was shaken for 2 min and left to stand for 2 h to remove any bubbles. The sieving matrix was made by dissolving 0.3% (w/v) of M_r 8,000,000 poly (ethyleneoxide) (PEO) (Sigma, St. Louis, MO, USA) into the 1× TBE buffer with 0.5 μg/ml EtBr, slowly stirring over night.

For the PCR analysis of soybean, PCR premix, Sapphire (components of 20 μl reaction: thermostable DNA polymerase 1 U, dNTPs 200 μM and $MgCl_2$ 1.5 mM) were purchased from Super-Bio (Suwon, Korea). A 100-bp DNA fragment from the GM soybean was amplified with forward primer for the CaMV 35S promoter, (35spF, 5′-TC GTTC AAGA TGCC TCTG CC-3′) and reverse primer, (35spR, 5′-TT GCTT TGAA GACG TGGT TGG-3′). A 250-bp DNA fragment from the GM soybean and the non-GM soybean were amplified with forward primer (Lec 250F, 5′-CT GACC AGCA AGGC AAAC TC-3′) and reverse primer (Lec 250R, 5′-GT GAAG TTGA AGGA AGCG GC-3′). All primers were synthesized by GenoTech (Daejen, Korea). DNA size markers, a 100-bp DNA ladder purchased from Genepia (Seoul, Korea) was diluted to 25 ng/μl with 1× TBE buffer before using.

2.2. PCR sample preparation

Soybean samples were acquired from the Bio-safety Research Institute at Chonbuk National University. The genomic DNA was extracted from 50 mg of soybean powder with or without GMO components through the CTAB (cetyltrimethyl-ammonium bromide) method [21,22]. Briefly, 600 μl of CTAB was added in an effendorf tube containing soybean powder and incubated at 55 °C for 2 h in 0.5 ml of extraction buffer (100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, 1.0% SDS, pH 8) containing proteinase K. Proteins were extracted with 500 μl of phenol/chloroform/isoamylalcohol (25/24/1, v/v/v). Subsequently, 5 μl of RNase (10 mg/ml) was added, and incubated at 37 °C for 30 min. This sample was spun down (12,000 rpm) in a micro-centrifuge for 5 min at room temperature, and the top layer was discarded. Next, 600 μl of cold 70% ethanol was added and spun down for 30 s (12,000 rpm). The liquid was pipetted off in a tube and the DNA pellet was washed once with 600 μl of cold 70% ethanol. Then, the DNA pellet was washed with 600 μl of absolute alcohol. For elution, a supplementary treatment was carried out with the addition of nuclease free water.

The PCR reaction was performed in a thermal cycler (MJ Research PTC-200, USA) using the following temperature protocol: 5 min incubation at 95 °C; 40 cycles of denaturing at 95 °C for 30 s, annealing at 57 °C for 1 min, extension at 72 °C for 30 s; followed by a 7 min hold at 72 °C for 100-bp DNA fragment and 250-bp DNA fragment. The 20 μl PCR reaction mixture had the following final composition: 10 μl of PCR premix, Sapphire, 0.5 μl each of forward and reverse primer, and 2 μl of purified DNA. Finally, each amplified PCR product was introduced into the slab gel electrophoresis system and the MCGE system, respectively.

2.3. Slab gel electrophoresis

Slab gel electrophoresis was performed in 2% agarose gel (Sigma, St. Louis, MO, USA) with TAE buffer. One microliter of 6× gel loading dye (bromo phenol blue: xylene cyanol FF: glycerol = 0.25:0.25:30, w/v(%)) was mixed with 5 μl of each specimen. Of this, 6 μl were loaded on four-well gels and run at 140 V for 60 min in SaB-Cell (Bio RAD, USA). After electrophoresis, the gel was stained with EtBr (0.5 μg/ml) for 5 min and de-stained in nuclease free water. Then, the samples were photographed over UV-light of Gel Doc 2000 (Bio RAD, USA) and visualized with EtBr. The presence of both a 250- and 100-bp band was recorded as positive results. Sizes of the DNA products were determined relative to those of size markers, 100 bp DNA ladder.

2.4. Microchip capillary gel electrophoresis

MCGE was performed on a DBCE-100 Microchip CE system (Digital Bio Technology Co., Korea) equipped with a diode-pumped solid-state laser (exciting at 532 nm and collecting fluorescence at 605 nm; Power Technology Inc., Little Rock, AZ, USA) and a high-voltage device (DBHV-100, Digital Bio Technology Co., Korea). The microchip, schott borofloat glass, was purchased from Micralyne (MC-BF4-TT100, Micralyne, USA). The injection design was a double-T channel with a 100-μm offset (Fig. 1). The chip channel was 50-μm wide and 20-μm deep. The reservoirs were 2.0 mm in diameter and 1-mm deep. The injection channel length (from reservoir 2 to reservoir 4) was 8.0 mm. The separation channel (from reservoir 1 to reservoir 3) was 85-mm long and detection was performed at 15 mm and 20 mm from the injection-T. The MCGE run buffer was 1× TBE buffer with 0.5 μg/ml of EtBr. The coating matrix and the sieving matrix were 0.5% PVP (M_r 1,000,000) and 0.3% PEO (M_r 8,000,000) for the 1× TBE buffer with 0.5 μg/ml EtBr, respectively. The sieving matrix was hydrodynamically filled by applying a vacuum of 8.67×10^4 Pa (EYELA A-3S vacuum aspirator, TOKYO RIKAKIKAI Co., Japan) to the MCE reservoir 3 for 3 min. The sample was pipetted into the sample inlet reservoir 2 of the microchip. The DNA sample injection via a conventional electrokinetic injection was conducted in the injection-T region by applying a potential of 480 V at sample outlet reservoir 4 followed by grounding the sample inlet reservoir 2 for 60 s. The applied voltage was in the range of 1.0–4.0 kV at the buffer inlet (1) and sample outlet (4). After each run, the microchip channel was rinsed in the following sequence; water and run buffer for 10 min each.

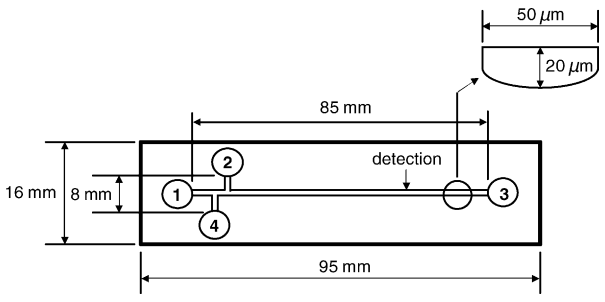

Fig. 1. Layout of the double-T microfluidic chip used for the separation of GMO PCR product. Reservoir 1 = buffer inlet, reservoir 2 = sample outlet, reservoir 3 = buffer waste, reservoir 4 = sample inlet.

2.5. Programmed field strength gradients

According to our previous paper [19], the PFSG separation was optimized as follows: first, we tried to find the constant electric field strength in the range of 100–500 V/cm for the separation of all DNA fragments of the DNA ladder. From the separation, decide whether PFSG or constant strength (i.e., staircase field strength) is best. If PFSG is chosen, eliminate or decrease the portions of the gradient prior to the first DNA peak (100-bp DNA) and following the last DNA peak (300-bp DNA). Finally, if the separation in the second step is acceptable, try reducing the gradient time to reduce the run time. The PFSG was programmed to give the best separation of all target DNA fragments (100- and 250-bp DNA) with resolutions >1.5.

3. Results and discussion

We analyzed soybean samples with or without GMO by PCR with the CaMV 35S promoter. The PCR amplified GM-soybeans and non GM-soybeans were analyzed with 2% agarose gel electrophoresis (Fig. 2). The results showed that the 100-bp DNA fragment representing the CaMV 35S promoter (lane 3 in Fig. 2) from GM soybeans and 250-bp DNA fragment representing the lectin endogenous gene present in GM or non-GM soybeans were amplified (lane 2 in Fig. 2). This indicated that the PCR condition was able to specifically amplify the GMO component (i.e., 100-bp DNA) in both the GM-soybeans and non GM-soybeans.

To evaluate the use of the MCE system (DBCE-100 Microchip CE system) for detecting and quantifying GMO, the PCR amplified product was used and the results were compared to traditional slab gel electrophoresis. At the constant electric field strength of 117.6 V/cm, the GM-soybean was successfully analyzed within 135 s in a 0.3% PEO (M_r 8,000,000) sieving matrix and 1× TBE run buffer (Fig. 3).

In general, an increase in electric field strength increased the velocity of DNA fragments and reduced migration time, leading to shorter analysis times (Fig. 4). This suggests that one should use the highest voltage available to the instrument for the most rapid analysis of DNA fragments. However, higher voltages lead to higher currents and increased Joule heating. Therefore, higher voltage and the resulting higher current cause an increase in heat production. Increased heat in the microchip may lead to broader peaks, non-reproducible migration times, sample decomposition or even boiling of the buffer, which can cause electrical discontinuity through-

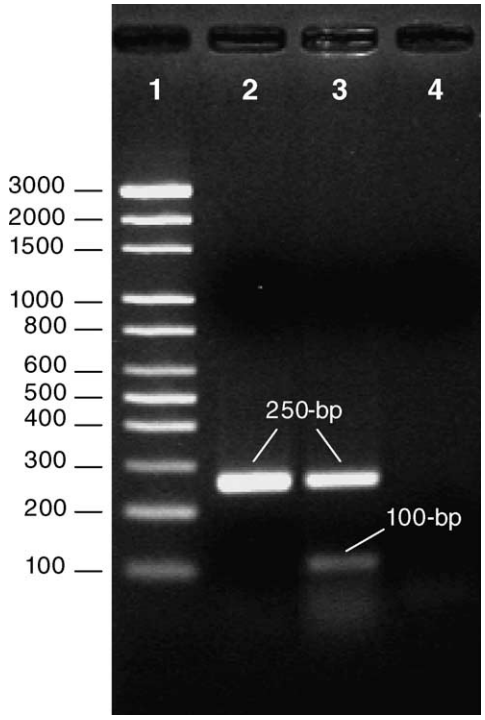

Fig. 2. Slab gel electrophoresis of PCR amplified GM-soybean and non GM-soybean. Lane 1, 100-bp DNA ladder; lane 2, non GM-soybean; lane 3, GM-soybean; lane 4, nuclease free water. Gel electrophoresis conditions: 2% agarose gel matrix in 1× TAE buffer; applied voltage, 140 V for 60 min; ambient temperature.

out the channel, shutting down the MCGE system and/or decrease in resolving power and efficiency.

Using a relatively high electric field, DNA molecules can be separated within a relatively short time. However, excessively high electric field strength caused the loss of resolving power in DNA fragments for the MCGE system (Table 1).

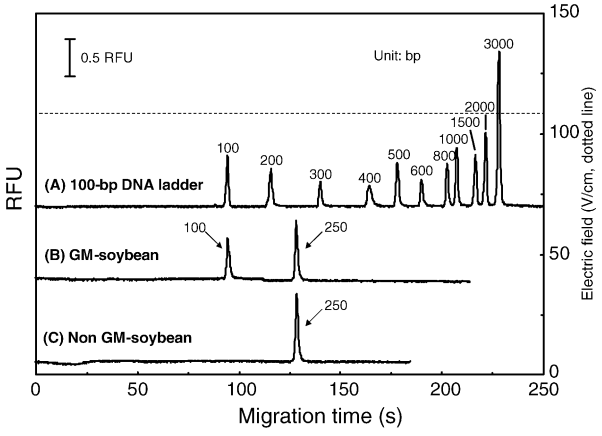

Fig. 3. MCGE separation of PCR products, 100- and 250-bp DNA fragments by constant field strength. Applied separation voltage conditions, 117.6 V/cm; electrokinetic injection, 0.48 kV for 60 s; run buffer, 1× TBE buffer (pH 8.3) with 0.5 ppm EtBr; coating matrix, 0.5% PVP (M_r 1,000,000); sieving matrix, 0.3% PEO (M_r 8,000,000); sample, PCR amplified 100-bp DNA ladder, GM-soybean and non GM soybean; *RFU: relative fluorescence unit.

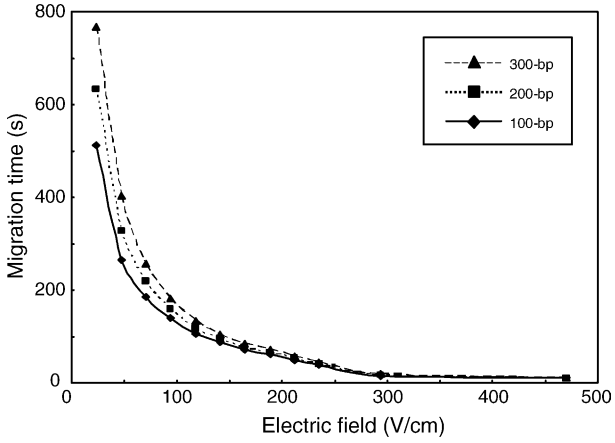

Fig. 4. Migration time of DNA fragments as a function of the applied electric field. MCGE voltage conditions; applied separation voltage, from 23.5 to 235.3 V/cm; sample, 100-bp DNA ladder fragment (100-, 200- and 300-bp); the running buffer, 1× TBE buffer (pH 8.3) with 0.5 (g/ml EtBr; coating matrix, 0.5% PVP (M_r 1,000,000); sieving matrix, 0.3% PEO (M_r 8,000,000).

Generally, the main separation mechanism in MCGE is based on differences in DNA size as analytes migrate through the pores of the gel-filled microchip. Small DNA molecules are able to pass through the pores and elute first, whereas larger DNA molecules are retarded by the gel and elute later. However, the chain entanglement also plays a significant role in the separation of DNA fragments with different chain lengths in a gel of a given pore size [24]. The entanglement is a function of the molecule size and the applied electric field [25]. Therefore, the electrophoretic mobility of DNA molecules becomes field-dependent [19,26] and the resolution showed irregular values.

Higher electric field strength yields shorter analysis times and decreases the resolving power of greater than 800-bp

Table 1
Resolutions between 100 and 300 bp DNA fragments under specific electric field

Electric field (V/cm)	Resolution (R_s)[a]	
	100- and 200-bp	200- and 300-bp
23.5	2.4241 (±0.2119)	2.4745 (±0.3080)
47.1	2.6341 (±0.2485)	2.9206 (±0.3655)
70.6	3.1634 (±0.3859)	3.0171 (±0.3593)
94.1	2.9240 (±0.4552)	3.2080 (±0.5827)
117.6	5.3365 (±0.3542)	3.6052 (±0.4152)
141.2	4.1682 (±0.4821)	4.2684 (±0.5130)
164.7	3.1737 (±0.4389)	3.6414 (±0.6378)
188.2	3.0449 (±0.5996)	3.4987 (±0.4386)
211.8	2.9223 (±0.5860)	3.4084 (±0.3482)
235.3	2.1960 (±0.5624)	2.2541 (±0.3421)
294.1	1.8333 (±0.2451)	1.7524 (±0.3073)
470.6	1.4619 (±0.2597)	1.3835 (±0.2429)

Applied separation voltage, from 0.2 to 2.0, 2.5 and 4.0 kV; effective length, 20 mm; coating matrix, 0.5% PVP (M_r 1,000,000); sieving matrix, 0.3% PEO (M_r 8,000,000).

[a] $R_s = \Delta t / w_{ave}$ (Δt: the difference of migration time of two adjacent peaks; w_{ave}: average peak width of baseline).

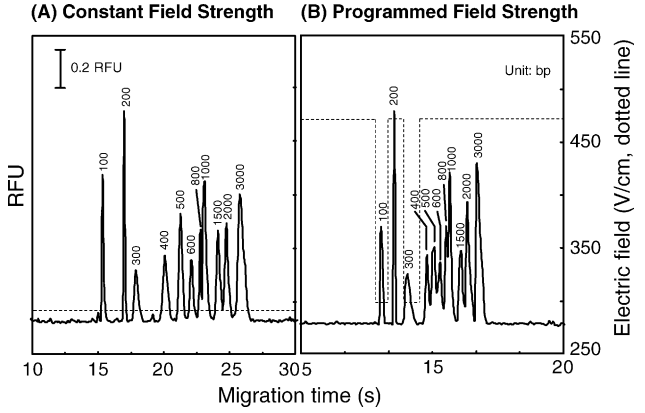

Fig. 5. Microchip capillary gel electrophoresis of 100-bp DNA ladder fragments under: (A) the constant high field strength and (B) the programmed field strength gradient method. MCGE condition: coating matrix, 0.5% PVP (M_r 1,000,000); sieving matrix, 0.3% PEO (M_r 8,000,000); applied separation (A) constant voltage, 294.1 V/cm, (B) applied separation voltage, 470.6 V/cm for 9 s, 294.1 V/cm for 1 s, 470.6 kV for 0.5 s, 294.1 V/cm for 1.5 s and 470.6 V/cm for 20 s. The dotted line represents the applied electric field.

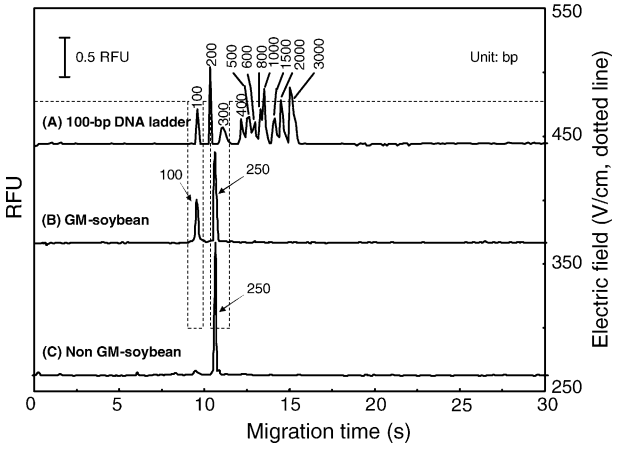

Fig. 6. MCGE separation of PCR products, 100- and 250-bp DNA fragments by programmed field strength gradient. 100-bp DNA ladder; running buffer, 1× TBE buffer (pH 8.3) with 0.5 μg/ml EtBr; coating matrix, 0.5% PVP (M_r 1,000,000); sieving matrix, 0.3% PEO (M_r 8,000,000); injection 60 s at 0.48 kV; applied separation voltage, 470.6 V/cm for 9 s, 294.1 V/cm for 1 s, 470.6 kV for 0.5 s, 294.1 V/cm for 1.5 s and 470.6 V/cm for 20 s.

DNA fragments in CE [27]. However, even though DNA molecules have the size of 100–300 bp, the resolving power of DNA fragments decreased at an electric field greater than ∼120 V/cm (Table 1). Therefore, determining the optimum electric field strength is the best approach to ensure the rapid detection of GM-soybeans in a MCGE without loss of resolving power. In order to show the effect of the electric field strength on resolution, the resolution of a critical pair of 100-bp DNA ladders (DNA size marker) was measured at different levels of electric field strength such as constant high electric field (Fig. 5A) and a programmed field strength gradient (Fig. 5B). When the electric field strength of 117.6 V/cm (Fig. 3) was increased to the constant high voltage of 294.1 V/cm (Fig. 5A), the separation time of DNA fragments decreased from 135 to 27 s. However, the resolving power of long DNA fragments (>600 bp) was significantly reduced. Another approach to decreasing the separation time of specific sizes of DNA fragments is to program the field strength to change during analysis, that is, use a programmed field strength gradient. Different electric fields are optimum for specific sized DNA fragments, so the PFSG can be programmed to provide the best separation of GM-soybean analysis (Fig. 5B).

With the separation PFSG such as 470.6 V/cm for 9 s, 294.1 V/cm for 1 s, 470.6 kV for 0.5 s, 294.1 V/cm for 1.5 s and 470.6 V/cm for 20 s, the GM-soybean was only analyzed within 11 s (Fig. 6). GM-soybean was analyzed about 12-times faster than using the simple constant field strength method without loss of resolution (Fig. 3B versus Fig. 6B). At the optimum MCGE-PSEG, the GM-soybean peak (100-bp) showed the percent relative uncertainties of 0.4% for the migration time and 2.3% for the peak area ($n = 5$), respectively. The lectin (250-bp) showed the migration time of 10.6934 (±0.0724) s at the 97% confidence interval. This demonstrates that the MCGE-PFSG method can be used to determine GMO content with a reasonable level of precision.

4. Conclusions

The enhanced and more rapid separation of GM-soybean can be achieved by applying a non-uniform FSG, PFSG. In the MCGE-PFSG, the electric field strengths at the beginning and end of the gradient played a major role in determining the adequacy of the applied electric field and the fast separation of specific sized DNA fragments.

Compared to MCGE using conventional and constantly applied electric field (isoelectrostatic), the MCGE-PFSG was presented a much faster analysis time (∼11 s) without loss of resolving power for a specific DNA fragment (100- and 250-bp DNA). This method can also be used for the ultra-fast analysis of GMOs in others foodstuffs without any design modification of the microchip and considering the DNA fragment size.

Acknowledgements

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References

[1] M. Dröge, A. Pühler, W. Selbitschka, J. Biotechnol. 64 (1998) 75.
[2] R.S. Hails, Trends Ecol. E 15 (2000) 14.
[3] B.E. Erickson, Anal. Chem. 72 (2000) 454A.

[4] L. Bonfini, P. Heinz, S. Kay, G. van den Eede, Food Products and Consumer Goods Unit, Ispra (VA), Italy, 2002, p. 1.
[5] F.E. Ahmed, Trends Biotechnol. 20 (2002) 215.
[6] P.J. Obeid, T.K. Christopoulos, P.C. Ioannou, Electrophoresis 25 (2004) 922.
[7] G. Liu, W. Su, Q. Xu, M. Long, J. Zhou, S. Song, Food Control 15 (2004) 303.
[8] G.M. Brett, S.J. Chambers, L. Huang, M.R.A. Morgan, Food Control 10 (1999) 401.
[9] H.R. Permingeat, M.I. Reggiardo, R.H. Vallejos, J. Agric. Food Chem. 50 (2002) 4431.
[10] A. Holst-Jensen, S.B. Ronning, A. Lovseth, K.G. Berdal, Anal. Bioanal. Chem. 375 (2003) 985.
[11] V. Garcia-Canas, R. Gonzalez, A. Cifuentes, J. Agric. Food Chem. 50 (2002) 1016.
[12] V. Garcia-Canas, R. Gonzalez, A. Cifuentes, J. Agric. Food Chem. 50 (2002) 4497.
[13] A. Manz, N. Graber, H.M. Widmer, Sens. Actuator B Chem. 1 (1990) 244.
[14] D.J. Harrison, A. Manz, Z. Fan, H. Lüdi, H.M. Widmer, Anal. Chem. 64 (1992) 1926.
[15] L. Zhang, F. Dang, Y. Baba, J. Pharm. Biomed. Anal. 30 (2003) 1645.
[16] A.T. Woolley, R.A. Mathies, Anal. Chem. 67 (1995) 3676.
[17] D. Schmalzing, A. Adourian, L. Koutny, L. Ziaugra, P. Matsudaria, D. Ehrlich, Anal. Chem. 70 (1998) 2303.
[18] C. Backhouse, M. Caamano, F. Oaks, E. Nordman, A. Carillo, B. Johnson, S. Bay, Electrophoresis 21 (2000) 150.
[19] S.H. Kang, M. Park, K. Cho, Electrophoresis, in press.
[20] P.N. Benfey, N.-H. Chua, Science 250 (1990) 959.
[21] R. Meyer, Food Control 10 (1999) 391.
[22] M. Park, H. Lee, S.H. Kang, J. Korean Chem. Soc. 49 (2005) 255.
[23] L. Birch, C.L. Archard, H.C. Parkes, D.G. McDowell, Food Control 12 (2001) 535.
[24] D.L. Smisek, D.A. Hoagland, Science 248 (1990) 1221.
[25] T. Demana, M. Lanan, M.D. Morris, Anal. Chem. 63 (1991) 2795.
[26] D.H. Flint, R.E. Harrington, Biochemistry 11 (1972) 4858.
[27] A. Guttman, B. Wanders, N. Cooke, Anal. Chem. 64 (1992) 2348.